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Phenylalkanoid glycosides (non-salicinoids) from wood chips of *Salix triandra* × *dasyclados* hybrid willow

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Abstract: *Salix triandra* (almond leaved willow) is an established crop, grown in coppicing regimes for basket-making materials. It is known as a source of non-salicinoid phenolic glycosides such as triandrin and salidroside. A spontaneous natural hybrid of *S. triandra* and *S. dasyclados* was subjected to metabolite profiling by high resolution LC-MS and 22 phenolic glycosides, including 18 that are new to the Salicaceae were identified. Structures were determined by HPLC isolation and NMR methods. The hybridisation process has introduced novel chemistry into the Salix phenolic glycoside palette, in particular the ability to generate disaccharide conjugates where the glycosyl group is further extended by a range of sugars including apiose, rhamnose, xylose and arabinose. Also of note is the appearance of chavicol derivatives, also not previously seen in *Salix* spp. The work demonstrates the plasticity of the phenolic glycoside biosynthetic pathway and the potential to improve established crops such as *S. triandra* and *S. dasyclados, via* high-value metabolites, for both basketry and bioenergy markets.

Keywords: phenylalkanoids; phenolic glycosides; *Salix triandra; Salix dasyclados;* chavicol; rosarin;

1. Introduction

The Salicaceae family is a distinct taxon of perennial woody, dioecious shrubs and trees that can primarily be divided into two genera: willows (*Salix* spp.) and poplars (*Populus* spp.). Both groups have a rich secondary chemistry based on phenolic glycosides. The genus *Salix* is the largest and includes over 400 species that are variable in growth form, from large trees to small shrubs, distributed over a wide range of habitats [1]. Willow bark preparations have been used for the treatment of fever and pain since ancient times [2] and these bioactivities have mostly been related to their constitutive salicinoids, which are defined as derivatives of salicyl alcohol with β-D-glucopyranose moieties (e.g. salicin and salicortin) [3]. Although salicinoids are the most commonly studied class of secondary metabolites in the Salicaceae, other phenolic glycosides as well as lignans, flavonoids, and terpenes have been characterised [4]. Considering the variety of compounds described in willows, the beneficial effects of herbal products may not be ascribed only to salicinoids. Hydroxycinnamic acid and benzoic acid derivatives, for example, have potential antioxidant, antimicrobial and anticancer activities. These

and other mono- and 1,4-di-substituted benzenoids may add value to short rotation coppice (SRC) willow crops[5], not only for such biomedical uses but also, if present in sufficient quantity, as feedstocks for aromatic bio-refining streams.

One of the best known species of willow that is not rich in salicinoids is *S. triandra* L. (almond-leaved willow) [6, 7]. This straight-stemmed shrubby species is commonly grown in plantations that are coppiced annually and used for basketry, and varieties such as 'Black Maul', which can grow 2-3m/year are major contributors to this industry. As a part of our ongoing research towards adding value to willow crops we are surveying *S. triandra* species contained in the 1500+ UK National Willow Collection (NWC), maintained as a short-rotation coppice plantation at Rothamsted Research, Harpenden, UK. In this paper, we report on the secondary metabolite profile of wood-chips of an unusual *Salix triandra* × *dasyclados* hybrid (*S.* × *schaburovii* I.Beljaeva) (NWC1283). This naturally occurring hybrid, originates from the flood plain of the Kama River, Perm, Russia and came to the NWC in 2004 from the Botanical Gardens of the Urals. The study builds on our previous work [8, 9] in the analysis of secondary metabolites in willow, particularly in the polar metabolites obtained from aqueous alcohol extractions [10].

2. Results and Discussion

Freeze-dried wood chips derived from the multiple whole stems of *S. triandra* × *dasyclados* (NWC 1283) grown in the field and harvested at the dormant stage (January), were extracted with aqueous ethanol and analysed by uHPLC–HRMS. The total ion chromatogram (**Figure 1**) showed that this extract is a complex mixture of compounds and, unlike most willow extracts, is not dominated by salicinoids, i.e. salicin is a relatively minor peak while salicortin, normally appearing at 20.5 min, is now totally obscured by more abundant peaks.

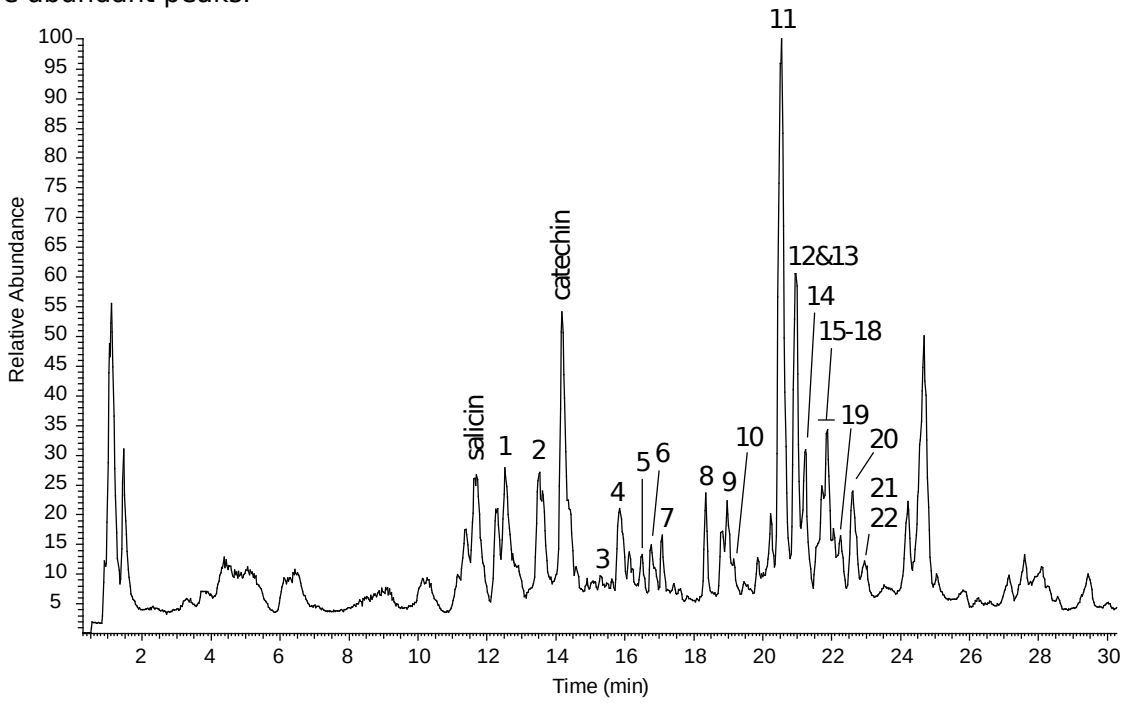

Figure 1. Total ion chromatogram (negative ion mode) extract generated from *Salix triandra* × *dasyclados* hybrid (NWC1283) following extraction in water:ethanol (4:1).

Peaks **1**, **2**, **11** and **14** were identified as picein, salidroside, rosarin and rosavin, respectively, in comparison with ^{1}H-NMR and LC-MS data (**Table 1**) of authentic standards run under identical conditions. Picein (**1**) has been detected in different parts of *Salix* species, such as leaves of *S. matsudana* [11] and bark of *S. purpurea* [12]. Salidroside (**2**) is also a major component in leaves of *S. triandra* and of its hybrids with *S. viminalis* and *S. purpurea* [13].

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Table 1. Phenylalkanoid glycosides identified in the hydroalcoholic extract of *Salix triandra* × *dasyclados* (NWC1283) wood chips.

No.	[*M*-H]- (*m/z*)	t_R (min)	Formula	Δ (ppm)	MS/MS ions (*m/z*)	Compound
1	297.0981	12.6	$C_{14}H_{18}O_7$	+0.55	**135**, 181	Picein[a]
2	299.1136	13.4	$C_{14}H_{20}O_7$	-0.03	119, **137**, 179	Salidroside[a]
3	325.0930	15.2	$C_{15}H_{18}O_8$	+0.29	89, 119, 145, **163**, 193	*p*-Coumaroyl- β-D-glc*p*[b]
4	311.1133	15.8	$C_{15}H_{20}O_7$	-1.11	**149**, 161	Triandrin[b]
5	325.1289	16.3	$C_{16}H_{22}O_7$	+0.47	**163**	p-Hydroxybenzylacetone-β-D-glc*p*[b]
6	401.1453	16.7	$C_{18}H_{26}O_{10}$	+0.01	161, **269**	Benzyl-β-D-api*f*-(1→6)-β-D-glc*p*[b]
7	401.1454	17.1	$C_{18}H_{26}O_{10}$	+0.31	161, **269**	Benzyl-β-D-xyl*p*-(1→6)-β-D-glc*p*[b]
8	415.1611	18.3	$C_{19}H_{28}O_{10}$	+0.31	179, **191**, 283	2-phenylethyl-α-L-ara*f*-(1→6)-β-D-glc*p*
9	415.1612	18.9	$C_{19}H_{28}O_{10}$	+0.46	**149**, 179, 191, 283	2-Phenylethyl-α-L-ara*p*-(1→6)-β-D- glc*p*[b]
10	415.1610	19.0	$C_{19}H_{28}O_{10}$	+0.07	89, **149**, 191, 283	2-Phenylethyl-β-D- api*f* -(1→6)-β-D-glc*p*[b]
11	427.1611	20.5	$C_{20}H_{28}O_{10}$	+0.24	125, 133, 149, **161**, 191, 293	Rosarin[a]
12	429.1768	20.9	$C_{20}H_{30}O_{10}$	+0.32	101, 131, 161, **297**	Dihydrorosarin[b]
13	341.1242*	21.0	$C_{15}H_{20}O_6$	+0.04	**133**, 161	Rosin[b]
14	427.1610	21.2	$C_{20}H_{28}O_{10}$	+0.09	125, 133, 149, **161**, 191, 233, 293	Rosavin[a]
15	429.1766	21.5	$C_{20}H_{30}O_{10}$	+0.03	101, 131, 161, **297**	Phenylpropanol-β-D- api*f* -(1→6)-β-D- glc*p*[c]
16	429.1767	21.6	$C_{20}H_{30}O_{10}$	+0.11	101, 131, 161, **297**	Dihydrorosavin[b]
17	441.1768	21.8	$C_{21}H_{30}O_{10}$	+0.52	101, **125**, 163, 247, 307	Cinnamrutinose A[b]
18	427.1611	21.9	$C_{20}H_{28}O_{10}$	+0.38	89, 125, **133**, 191, 233, 293	Chavicol-α-L- ara*f* -(1→6)-β-D-glc*p*[b]
19	427.1610	22.1	$C_{20}H_{28}O_{10}$	+0.02	89, 125, 133, **149**, 191, 233, 293	Chavicol-α-L- ara*p* -(1→6)-β-D-glc*p*[c]
20	427.1610	22.6	$C_{20}H_{28}O_{10}$	+0.09	89, 125, 133, 149, 191, 233, **293**	Chavicol-β-D- api*f* -(1→6)-β-D-glc*p*[b]
21	341.1243*	22.9	$C_{15}H_{20}O_6$	+0.31	**133**, 161	Chavicol-glucoside[b]
22	441.1766	23.0	$C_{21}H_{30}O_{10}$	-0.03	101, **125**, 163, 247, 307	Chavicol-rutinoside[b]

* corresponds to the formate adduct.
[a] Identification based on comparison with standards.
[b] Identification based on isolation of the compounds and NMR.
[c] Tentative identification based on the detection of some of its NMR and LC-HRMS signals as impurities of other compounds.
Abbreviations, glc*p*: glucopyranoside, api*f*: apiofuranosyl, xyl*p*: xylopyranosyl, ara_f: arabinofuranosyl, ara*p* arabinopyranosyl.

In order to identify further peaks, a portion of the extract was fractionated by semi-preparative HPLC via repeated injections After solvent removal, these fractions were analysed by NMR. In total twenty-two phenylalkanoid glycosides were identified on the basis of the obtained HRMS and/or NMR data compared with those found in the literature. **Table 1** includes uHPLC–HRMS data and the method of identification for these

compounds, while their chemical structures can be found in **Figure 2**. Nineteen of the identified compounds are described for the first time in a *Salix* genotype and one of them (**16**) is a novel molecule.

Peak **3** at 15.2 min with $[M–H]^-$ at *m/z* 325.0930 had the molecular formula of $C_{15}H_{18}O_8$ and was identified as *p*-coumaroyl-β-D-glucopyranoside based on its ^{1}H-NMR peaks (**Table 2**) [14]. The molecule contained a pair of aromatic doublets, each with an 8.6 Hz coupling, at δ6.96 and 7.60 indicating a para substituted molecule. A β-glucoside moiety was confirmed *via* inspection of the anomeric signal which appeared as a doublet at δ5.65 and had an 8 Hz coupling. Peak **4** at 15.8 min corresponded to the known compound triandrin, in which *m/z* 357.1187 arises from its formate adduct and 311.1133 its molecular ion [15]. Triandrin (**4**) has been recently found in the leaves and twigs of *S. reticulata* [16], but is better known in *S. triandra* [6, 7], a parent of NWC1283. Compound **5** eluted at 16.3 min and showed *m/z* 371.1347 $[M-H+HCOOH]^-$. Comparison of its NMR data to those of a compound isolated from the roots of *Rheum officinale* Baill. (Polygonaceae) confirmed it to be *p*-hydroxybenzylacetone-β-D-glucopyranoside [17] (**Table S1**).

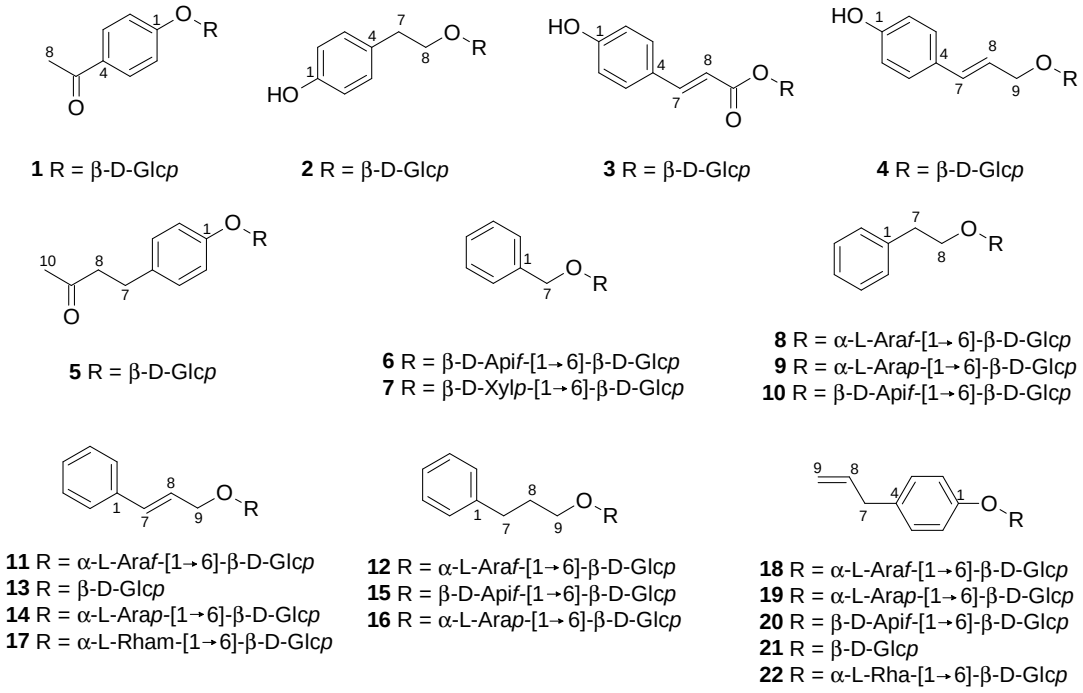

Figure 2. Chemical structures of phenylalkanoid glycosides identified in the hydroalcoholic extract of *S. triandra* × *dasyclados* hybrid (NWC1283) chipped biomass.

Compound **21** eluted at 22.9 min with *m/z* at 341.1243 corresponding to the formate adduct of the molecular formula $C_{15}H_{20}O_6$. The ^{1}H-NMR spectrum of this compound presented aromatic (δ_H 7.07 and 7.23 ppm, *d*, J=8.7 Hz) and anomeric (δ_H 5.04 ppm, *d*, J=7.7 Hz) signals (**Table 2**) similar to those of compound **5**. This indicates that this molecule contains a *para*-disubstituted aromatic ring and that the glucose moiety is *O*-linked to this ring. In addition to it, signals of an allylic side chain (terminal olefinic proton at δ 5.08 ppm; multiplet at δ 6.01 ppm and doublet at δ 3.35 ppm) were also detected and the structure of **21** was confirmed to be chavicol-β-D-glucopyranoside, a compound previously isolated from *Cedronella canariensis* (L.) Webb & Berth. (Lamiaceae) [18] and *Alpinia officinarum* Hance (Zingiberaceae) [19].

A further four peaks can be assigned to chavicol with a diglycosidic chain. Compounds **18** and **20** were isolated and their MSMS data of both compounds showed ions at m/z 133, corresponding to the loss of a hexose-pentose fragment (m/z 295). In **18**, two sugar moieties were confirmed from ^{1}H and ^{13}C NMR data of the anomeric

protons (δ_H/δ_C 5.04/103.5 and 5.00/111.2)(**Table 3** and **Table S2**). A coupling constant of 7.7 Hz for the anomeric signal at δ 5.04 suggested a β-glucoside linkage to the chavicol whilst the smaller (1.3 Hz) coupling of the anomeric signal at δ5.00 indicated an α-linkage of the pentose entity. ^{1}H-NMR signals agreed well with the published literature data for chavicol-α-L-arabinofuranosyl- (1→6)-β-D-glucopyranoside [20]. The furanosyl form of the arabinose moiety was confirmed by the downfield shift of the 1″-anomeric proton, appearing at δ5.00 and the ^{13}C resonance at δ111.2 [21]. Chavicol-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside was the structure assigned to **20**. This was confirmed by the coalescence of the 5″-H_2 signals to a singlet, appearing at δ3.60, and characteristic of the apiose moiety. In addition, a quarternary signal was evident from the ^{13}C-NMR data at δ82.3 (**Table 3** and **Table S2**). Both **18** and **20** have previously been found in *Betula papyrifera* Marsh. (Betulaceae) [20] and the NMR data was consistent for both compounds. Detailed ^{1}H and ^{13}C NMR spectroscopic data, including 2D correlations, can be found in **Table S2**. Peak **19,** with the same molecular formula ($C_{20}H_{28}O_{10}$),was putatively annotated as chavicol-α-L-arabinopyranosyl- (1→6)-β-D-glucopyranoside due to its proximity to **18** in the chromatographic run (less than 0.2 min later) and that **19** is an impurity in the ^{1}H-NMR spectrum of **18**, being detected by the anomeric proton of the arabinopyranosyl moiety (δ_H 4.46 ppm, *d*, J=8.0 Hz). The fourth compound eluted at 23.0 min with *m/z* at 441.1766 and was identified as chavicol-rutinoside (**22**) [19].

Table 2. ^{1}H-NMR data of monosaccharides found in *S. triandra* × *dasyclados* hybrid (NWC1283) wood chip extracts.

Position	(**1**)	(**2**)	(**3**)	(**4**)	(**5**)	(**13**)	(**21**)
1	-	-	-	-	-	-	-
2	7.22 *d* (8.9)	6.84 *d* (8.6)	6.93 *d* (8.6)	6.88 *d* (8.6)	7.07 *d* (8.6)	7.52 *d* (7.4)	7.07 d (8.7)
3	8.03 *d* (8.9)	7.21 *d* (8.6)	7.60 *d* (8.6)	7.41 *d* (8.6)	7.23 *d* (8.6)	7.41 *d* (7.6)	7.23 *d* (8.7)
4	-	-	-	-	-	7.34 *d* (7.5)	-
5	8.03 *d* (8.9)	7.21 *d* (8.6)	7.60 *d* (8.6)	7.41 *d* (8.6)	7.23 *d* (8.6)	7.41 *d* (7.6)	7.23 *d* (8.7)
6	7.22 *d* (8.9)	6.84 *d* (8.6)	6.93 *d* (8.6)	6.88 *d* (8.6)	7.07 *d* (8.6)	7.52 *d* (7.4)	7.07 *d* (8.7)
7	-	2.88 *t* (7.1)	7.84 *d* (15.9)	3.68 *d* (15.9)	2.86 *m*	6.77 *d* (16.3)	3.36 *d* (6.8)
8	2.64 *s*	4.08 dt (7.1, 10.1) 3.85 dt (7.3, 10.1)	6.48 *d* (15.9)	6.26 *dt* (15.9, 6.6)	2.88 *m*	6.42 *m*	6.01 *ddt* (17, 10.1, 6.8)
9	-	-	-	4.51 *ddd* (1.2, 6.2, 12.4)) 4.38 *ddd* (1.0, 7.0, 12.4)	-	4.55 *m*, 4.41 *m*	5.08 *d* (17.0)
10	-	-	-	-	2.19 s	-	-
1′	5.23 *d* (7.5)	4.44 *d* (8.0)	5.65 *d* (8.0)	4.52 *d* (8.0)	5.05 *d* (7.7)	4.53 *d* (8.0)	5.04 *d* (7.7)
2′	3.60-3.62 *m*	3.23 *dd* (8.0, 9.3)	3.62-3.46 *m*	3.29 *dd* (8.0, 9.3)	3.53 *d* (7.8)	3.49-3.39 *m*	3.59-3.41 *m*
3′	3.60-3.62 *m*	3.45 *d* (9.0)	3.62-3.46 *m*	3.47 *d* (8.9)	3.58 *d* (9.0)	3.49-3.39 *m*	3.59-3.41 *m*
4′	3.52 *d* (9.7)	3.39 *d* (8.9)	3.62-3.46 *m*	3.39 *d* (9.6)	3.47 *d* (9.0)	3.49-3.39 *m*	3.59-3.41 *m*
5′	3.67 *ddd* (2.2, 5.7, 9.8)	3.40 *ddd* (2.2, 9.8, 6.7)	3.62-3.46 *m*	3.41 *ddd* (2.1, 5.7, 9.8)	3.43 *m*	3.49-3.39 *m*	3.59-3.41 *m*
6′	3.94 *dd* (2.2, 12.5)	3.89 *dd* (2.2, 12.3)	3.79 *dd* (2.0, 12.0)	3.90 *dd* (2.1, 12.3)	3.92 *dd* (2.1, 12.4)	3.91 *dd* (2.1, 12.3)	3.90 *dd* (2.3, 12.4)

3.77 *dd* (5.7, 12.5)	3.70 *dd* (5.7, 12.3)	3.71 *dd* (5.2, 12.0)	3.72 *dd* (5.8, 12.3)	3.73 *dd* (5.8, 12.4)	3.72 *dd* (6.0, 12.2)	3.71 *dd* (5.6, 12.2)

Data collected in 80:20 D_2O:CD_3OD (4:1). Spectra were referenced to TSP-d_4 at δ0.00. Coupling constants in Hz are given in parentheses. Abbreviations, *s*: singlet, *d*: doublet, *t*: triplet, *dd*: doublet of doublets, *dt:* doublet of triplets, *ddd:* doublet of double doublets, *m*: multiplet

Monosubstituted aromatic glycosides were also identified in this study. Two compounds with molecular ions at *m/z* 401 (**6** and **7**) and three at *m/z* 415 (**8**–**10**) had their molecular formulas calculated for $C_{18}H_{26}O_{10}$ and $C_{19}H_{28}O_{10}$, respectively, based on accurate masses. These five compounds were purified for structural characterisation and NMR data identified them as benzyl-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**6**), benzyl-β-*D*-xylopyranosyl-(1→6)-β- *D*-glucoside (**7**), previously described in *Jasminum sambac* Ait. (Oleaceae) [22], 2-phenylethyl-α-L-arabinofuranosyl-(1→ 6)-β-D-glucopyranoside (**8**), 2-Phenylethyl-α-L- arabinopyranosyl-β-D-glucopyranoside (**9**) and 2-Phenylethyl-β-D-apiofuranosyl-β-D- glucopyranoside (**10**). Their ^{1}H NMR chemical shift data is presented in **Table 4** and **6**, **7**, **9** and **10** have been detected in the aerial parts of *Hylomecon vernalis* Maxim. (Papaveraceae) [23].

Peaks **11**, **13**, **14** and **17** corresponded to the cinnamyl alcohol derivatives rosarin, rosin, rosavin and cinnamrutinose A (**Table 2 and 4**). The first three compounds, together with salidroside (**2**), are commonly found in *Rhodiola rosea* L. (Crassulaceae) [24] and NMR data was in agreement with the published values. Cinnamrutinose A (**17**), on the other hand, has been isolated from a more taxonomically similar species, *Populus tremula* (Salicaceae) [25].

Compound (**12**) had $[M-H]^-$ at *m/z* 429.1767 corresponding to the molecular formula $C_{20}H_{30}O_{10}$. NMR data of this isolated compound was comparable to dihydrocinnamyl alcohol-α-L-arabinofuranosyl-(1→ 6)-β-D-glucopyranoside) (dihydro-rosarin) formerly described in *Juniperus communis* var. *depressa* [26]. Compound (**16**) showed the same molecular formula, however, NMR data indicated a different sugar moiety (**Table 5**). 1D ^{1}H NMR spectrum revealed the presence of a monosubstituted benzene ring, a hydroxypropyl group as well as two sugars, a β-glucopyranose (anomeric proton at δ_H 4.42/ δ_C 105.9) and a α-arabinopyranose (anomeric proton at δ_H 4.53/ δ_C 104.4). Long range correlations observed between H-7/C-1 (δ_H 2.71/ δ_C 145.3), H-1/C-9 (δ_H 4.42/ δ_C 72.8) in the HMBC spectrum indicated how each moiety was connected in the molecule. Thus, structure of **16** was determined to be dihydrocinnamyl alcohol-α-L-arabinopyranosyl-(1→6)-β-D- glucopyranoside (dihydro-rosavin) and this compound has not previously been reported in the literature.

Table 3. ^{1}H-NMR data of cinnamyl and chavicol disaccharides found in *S. triandra* × *dasyclados* hybrid (NWC1283) wood chip extracts.

Position	(**17**)	(**18**)	(**20**)	(**22**)
2	7.50 *d* (7.3)	7.07 *d* (8.7)	7.07 *d* (8.6)	7.07 *d* (8.6)
3	7.40 *d* (7.6)	7.22 *d* (8.7)	7.23 *d* (8.6)	7.23 *d* (8.6)
4	7.33 *d* (7.4)	-	-	-
5	7.40 *d* (7.6)	7.22 *d* (8.7)	7.23 *d* (8.6)	7.23 *d* (8.6)
6	7.50 *d* (7.3)	7.07 *d* (8.7)	7.07 *d* (8.6)	7.07 *d* (8.6)
7	6.74 *d* (16.0)	3.36 *d* (6.7)	3.36 *d* (6.9)	3.36 *d* (6.9)
8	6.39 *dt* (6.5, 16.0)	6.01 *ddt* (16.9, 10.1, 6.7)	6.01 *ddt* (16.9, 10.1, 6.7)	6.01 *ddt* (16.9, 10.1, 6.7)
9	4.50 *ddd* (12.7, 6.0, 1.2) 4.4 *ddd* (1.1, 6.8, 12.7)	5.07 *m*	5.08 *m*	5.08 *m*
1’	4.51 *d* (8.0)	5.04 *d* (7.7)	5.03 *d* (7.6)	5.03 *d* (7.6)
2’	3.46 *d* (9.1)	3.54 *d* (7.7)	3.53 *d* (7.6)	3.55-3.40 *m*
3’	3.45 *d* (9.1)	3.50 *d* (9.1)	3.56 *d* (8.9)	3.55-3.40 *m*

4’	3.41 *dd* (1.8, 9.6)	3.57 *d* (9.0)	3.48 *d* (9.2)	3.55-3.40 *m*
5’	3.53 *ddd* (1.8, 5.9, 9.6)	3.73 *m*	3.70 *m*	3.55-3.40 *m*
6’	3.97 *dd* (1.8, 11.6) 3.68 *dd* (5.9, 11.6)	4.04 *dd* (1.3, 11.1) 3.70 *m*	4.02 *dd* (2.3, 11.4) 3.72 *m*	4.12 *dd* (1.7, 11.9), 3.74 *m*
1’’	4.52 *d* (1.7)	5.00 *d* (1.3)	5.05 *d* (3.1)	5.07 *d* (1.8)
2’’	3.94 *dd* (1.8, 3.4)	4.06 *dd* (1.5, 3.3)	3.94 *d* (3.1)	4.09 *dd* (1.6, 3.3)
3’’	3.77 *dd* (3.4, 9.7)	3.89 *dd* (3.3, 5.9)	-	3.75 *m*
4’’	3.42 *d* (9.6)	4.00 td (3.3, 5.9)	3.83 *d* (10.1) 3.99 *d* (10.1)	3.40 *m*
5’’	3.72 *dd* (6.3, 9.6)	3.74 *dd* (3.3, 12.3) 3.64 *dd* (5.7, 12.3)	3.60 *s*	3.72 *dd* (6.2, 9.3)
6’’	1.27 *d* (6.3)	-	-	1.19 *d* (6.2)

Data collected in 80:20 D_2O:CD_3OD (4:1). Spectra were referenced to TSP-d_4 at δ0.00. Coupling constants in Hz are given in parentheses Abbreviations, *s*: singlet, *d*: doublet, *dd*: doublet of doublets, *ddt:* doublet of double triplets, *m*: multiplet

Although compound **15** could not be purified, the uHPLC–MS chromatogram of the fraction that contains **16** indicates that a small proportion of **15** can be detected in it (two peaks not well resolved, data not shown). In order to find more evidence of the structure of **15**, a careful analysis of 1D ^{1}H and 2D ^{1}H-^{13}C HSQC and HMBC NMR spectra of **16** was performed. Some minor peaks corresponding to a β-apiofuranosyl moiety were observed, such as an anomeric at δ_H 5.07 ppm (d, J=3.2 Hz)/ δ_C 111.9 ppm (CH-1’’); δ_H 3.96 ppm (d, J=3.2 Hz)/ δ_C 79.6 ppm (CH-2’’); δ_C 82.3 ppm (C-3’’); δ_H 4.00 ppm (d, J=10.1 Hz), 3.85 ppm (d, J=10.1 Hz)/δ_C 76.7 ppm (CH_2-4’’); and δ_H 3.62 ppm (s)/δ_C 66.5 ppm (CH_2-5’’). The relative integration of these peaks to the corresponding signals of **16** was 1:3. Together with this data, the fact that **15** presented the same molecular formula and mass fragments of **16** and the proximity of them in the uHPLC run (less than 0.2 min), we can suggest **15** is dihydrocinnamyl alcohol-β-apiofuranosyl-(1→ 6)-β-glucopyranoside.

Table 4. ^{1}H-NMR data of benzyl disaccharides found in *S. triandra* × *dasyclados* hybrid (NWC1283) wood chip.

Position	(**6**)	(**7**)	(**8**)	(**9**)	(**10**)
2	7.47 dd (1.6, 8.2)	7.46 *m*	7.40-7.37 *m*	7.39-7.36 m	7.39-7.36 m
3	7.44 dd (7.1, 1.6)	7.44 dd (1.5, 7.6)	7.37-7.33 *m*	7.36-7.34 m	7.36-7.34 m
4	7.41 d (7.0)	7.41 dd (1.6, 8.7)	7.29 *t* (7.3)	7.29 t (7.0)	7.29 t (7.0)
5	7.44 dd (7.1, 1.6)	7.44 dd (1.5, 7.6)	7.37-7.33 *m*	7.36-7.34 m	7.36-7.34 m
6	7.47 dd (1.6, 8.2)	7.46 *m*	7.40-7.37 *m*	7.39-7.36 m	7.39-7.36 m
7	4.91 d (11.7) 4.75 d (11.7)	4.94 d (11.7) 4.75 d (11.7)	2.97 *t* (7.0)	2.97 t (7.0)	2.97 t (7.0)
8	-	-	3.91 *m* 4.12 *dt* (6.2, 9.0)	3.91 m 4.13 m	3.91 m 4.13 m
1’	4.52 *d* (8.0)	4.52 d (7.9)	4.45 *d* (7.9)	4.45 d (8.0)	4.45 d (8.0)
2’	3.28 *m*	3.28 m	3.25 *d* (9.0)	3.23 d (8.0)	3.23 d (8.0)
3’	3.43 *m*	3.43 m	3.46 *d* (9.0)	3.45 m	3.45 m
4’	3.46 *d* (9.0)	3.46 m	3.41 *d* (9.4)	3.45 m	3.45 m
5’	3.53 ddd (2.0, 6.0, 9.0)	3.59 m	3.56 *dd* (2.0, 5.9)	3.39 dd (2.0, 9.0)	3.39 dd (2.0, 9.0)
6’	4.02 *dd* (2.0, 11.6) 3.73 *dd* (6.0, 11.6)	4.15 dd (1.9, 11.7), 3.85 dd (5.7, 11.7)	3.68 *dd* (5.8, 12.1) 4.03 *dd* (2.0, 12.1)	4.01 dd (1.9, 11.6), 3.70 dd (5.8, 11.5)	4.01 dd (1.9, 11.6), 3.70 dd (5.8, 11.5)
1’’	5.11 *d* (3.2)	4.45 d (7.8)	5.05 *d* (1.4)	4.44 d (8.0)	5.09 d (3.2)
2’’	4.01 *d* (3.2)	3.32 m	4.10 *dd* (1.5, 3.3)	3.30 m	3.98 d (3.2)

3''	-	3.42 m	3.92 *dd* (3.3, 5.7)	3.45 m	-
4''	4.06 *d* (10.1) 3.89 *d* (10.1)	3.59 m	4.05 *dd* (3.3, 5.9)	3.89 m	4.03 d (10.1) 3.87 d (10.1)
5''	3.66 *s*	3.30 m, 3.95 dd (5.4, 11.6)	3.80 dd (3.2, 12.2) 3.69 dd (5.0, 12.2)	3.89 dd (2.2, 12.3) 3.69 dd (5.0, 12.3	3.63 d (1.7)
6''	-	-	-	-	-

Data collected in 80:20 D_2O:CD_3OD (4:1). Spectra were referenced to TSP-d_4 at δ0.00. Coupling constants in Hz are given in parentheses. Abbreviations, *s*: singlet, *d*: doublet, *t*: triplet, *dd*: doublet of doublets, *dt:* doublet of triplets, *m*: multiplet

In summary, the hybrid of *S. triandra* and *S. dasyclados* (NWC1283) contains a much larger array of chemistry, particularly of phenylalkanoid disaccharides than has previously been reported in either of the parental species. Apart from salidroside (**2**) which was the only phenyethanoid glycoside previously known in the Salicaceae [27], many of the compounds reported have not been reported from willow before. Of particular note is the appearance of rosavin, rosarin and chavicol analogues and the propensity of these and others to contain disaccharide groups where the 'normal *Salix*' glycosyl moiety is further substituted at C-6 by arabinose, apiose, xylose or rhamnose. This second glycosidic substitution can also consist of either the pyranosyl form or of the furanosyl form and evidence of both forms is present for several of the compounds isolated. As evidenced in this study, such compounds can be isolated directly from chipped biomass with relatively straightforward extraction techniques. In this study, extraction was achieved using 20% aqueous ethanol, a solvent system that is cheap and therefore applicable to larger scale extractions. Similarly, heating steps were not required to release the compounds reported and extraction at room temperature preserved the integrity of the compounds. This work also demonstrates the potential of plant breeding to introduce traits that result in new chemistry, potential bioactivities and provide the opportunity to add value to basketry and bioenergy crops.

Table 5. NMR data of dihydro-rosarin **(12)** and dihydro-rosavin **(16)**.

	(12)				**(16)**			
Pos$_n$	δ_C [a]	δ_H [b]	COSY	HMBC	δ_C [a]	δ_H [b]	COSY	HMBC
1	145.3	-			145.3	-		-
2	131.6	7.30 *d* (7.0)	H-3/5, 4	C-4	131.6	7.31 *d* (7.1)	H-3/5, 4	C-3/5, 4, 7
3	131.5	7.35 *d* (7.5)	H-2/6, 4	C-1	131.5	7.35 *d* (7.5)	H-2/6, 4	C-1, 2/6
4	129.1	7.25 *t* (7.3)	H-2/6, 3/5	C-2/6	129.1	7.25 *t* (7.3)	H-2/6, 3/5	C-2/6
5	131.5	7.35 *d* (7.5)	H-2/6, 4	C-1	131.5	7.35 *d* (7.5)	H-2/6, 4	C-1, 2/6
6	131.6	7.30 *d* (7.0)	H-3/5, 4	C-4	131.6	7.31 *d* (7.1)	H-3/5, 4	C-3/5, 4, 7
7	34.3	2.71 *t* (7.6)	H-8	C-1, 2/6, 9	34.3	2.71 *t* (7.6)	H-8	C-1, 2/6, 8, 9
8	33.8	1.93 *dt* (6.7, 13.7)	H-7, 9	C-1, 9	33.8	1.93 *dt* (6.7, 13.7)	H-7, 9	C-1, 7, 9
9	72.8	3.88 *m* 3.65 *m*	H-8		72.8	3.89 *m* 3.65 *m*	H-8	
1′	105.5	4.41 *d* (7.9)	H-2'	C-9	105.9	4.42 *d* (7.4)	H-2'	C-9
2′	76.2	3.25 *d* (9.2)	H-1', 3'	C-3', 4'	76.2	3.28 *d* (9.0)	H-1', 3'	C-1', 3', 4'
3′	78.9	3.45 *m*	H-2'	C-2', 4'	79	3.45 *m*	H-2'	C-5'
4′	72.5	3.45 *m*	H-5'	C-3'	72.5	3.45 *m*	H-5'	C-5'
5′	72.7	3.41 *dd* (9.5, 2.5)	H4'	C-2', 3', 4', 6'	72.7	3.39 *dd* (9.0, 3.5)	H4'	C-3', 4'

6′	69.7	4.02 *dd* (11.5, 1.9) 3.68 *dd* (11.5, 6.0)	C-4', 5'	71.5	4.11 *dd* (1.9, 11.7) 3.82 *dd* (5.5, 11.7)		C-1'
1''	111.2	5.04 *d* (1.3)	C-6', 2'', C-3''	104.4	4.53 *d* (7.9)		C-2''
2''	87	4.02 *dd* (1.8, 3.6)	C-3''	72.1	3.58 *dd* (1.2, 5.4)		C-3''
3''	79.5	3.90 *dd* (6.2, 3.0)	C-4''	78.4	3.57 *d* (5.5)	H-4''	C-2''
4''	84	4.07 *dd* (1.6, 3.3)	C-3''	68.1	3.92 *d* (5.5)	H-3'', 5''	C-2'', 3''
5''	64.3	3.77 *dd* (12.3, 3.3) 3.66 *d*d (12.3, 5.0)	C-3''	63.7	3.89 *dd* (12.3, 2.3) 3.71 *dd* (12.3, 5.8)	H-4''	C-3''

Data collected in 80:20 D_2O:CD_3OD (4:1). Spectra were referenced to TSP-d_4 at δ0.00. Coupling constants in Hz are given in parentheses. [a] Measured at 150 MHz; [b] Measured at 600 MHz; Abbreviations, *s*: singlet, *d*: doublet, *t*: triplet, *dd*: doublet of doublets, *dt:* doublet of triplets, *m*: multiplet

3. Materials and Methods

3.1. General experimental procedures

^{1}H-1D and ^{1}H-^{1}H & ^{1}H-^{13}C 2D-NMR spectra of each compound were acquired, using a 5 mm triple resonance (TCI) cryoprobe, on a Bruker Avance 600 MHz NMR spectrometer (Bruker Biospin, Germany), operating at 600.05 MHz for ^{1}H and 150.9 MHz for ^{13}C NMR spectra. Typical 1-dimensional ^{1}H spectra were obtained with an acquisition time of 4.6 s, a sweep width of 7142.9 Hz and 65,536 data points. A total of 16 scans were recorded using the zgpr pulse sequence with a 90° angle. A relaxation delay of 5s was used to suppress the residual HOD signal. Spectra were transformed using an exponential window with a line broadening of 0.5 Hz. ^{1}H-^{1}H correlation spectroscopy (COSY) were run using the pulse sequence cosyprqf for 3 h and the frequency was 600.05 MHz in both dimensions. Acquisition times were 0.1434 and 0.0896 s and the sweep widths were 7,142.9 Hz. 1024 data points were collected in each dimension using 32 transients. ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra were performed using the pulse sequence hsqcetgpsi2 for 10 h, at 600.05 and 150.9 MHz frequencies with acquisition times of 0.1433 and 0.00212 s. Data were acquired using sweep widths of 7,142.9 and 30,120.5 Hz. 2048 and 1024 data points were collected using 128 transients. ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) spectra were obtained using the pulse sequence hmbcgpndqf for 22 h. Acquisition parameters were the same as stated for HSQC data collection.. For comparability with previous work [10], all spectra were collected at 300 °K in D_2O:CD_3OD (8:2) and chemical shifts are given in δ, relative to TSP-d_4 [(trimethylsilyl) propionic acid, 0.01 % w/v] added as a chemical shift reference standard. Compound concentration was typically 1 mg/mL. Phasing and baseline correction were carried out within TOPSPIN v. 2.1 (Bruker Biospin, Germany). Structural assignments of carbohydrate moieties were made with reference to authentic standards and the use of characteristic chemical shift data [21].

uHPLC–MS were recorded on an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher, Germany) coupled to a Dionex UltiMate 3000 RS uHPLC system, equipped with a DAD-3000 photodiode array detector. Separation was carried out in reverse-phase using Hypersil GOLD™ column (1.9 µm, 30 × 2.1 mm i.d. Thermo Fisher Scientific, Germany) which was maintained at 35 °C. The solvent system consisted of water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B), both Optima™ grade (Thermo Fisher Scientific, Germany). The injection volume was 10 µL and separation was carried out for 40 min with a flow rate of 0.3 mL/min under the following gradient: 0-5 min, 0% B; 5-27 min, 31.6% B; 27-34 min, 45% B; 34-37.5 min, 75% B. Mass spectra were collected in negative

ion mode using a heated electrospray source (Thermo Fisher Scientific, Germany). Resolution was 120,000 over *m/z* 50–1500 and the source voltage, sheath gas, auxiliary gas, sweep gas and capillary temperature were set to 2.5 kV, 35 (arbitrary units), 10 (arbitrary units), 0.0 (arbitrary units) and 350 °C, respectively.. Automatic MS–MS was performed on the four most abundant ions using an isolation width of *m/z* 2. Ions were fragmented with a normalised collision energy of 65 and an activation time of 0.1 ms, using high-energy C-trap dissociation . Data was collected and inspected using Xcalibur v. 2.2 (Thermo Fisher Scientific, Germany).

Compounds were isolated by repeated injection into an HPLC system (Dionex UltiMate 3000, Thermo Fisher Scientific) equipped with an Ascentis C-18 column (5 µm, 5 × 250 mm i.d., Supelco, UK). The column was maintained at 35 °C and chromatographic separation was performed using a constant flow rate of 1 ml/min, The mobile phases were water (A) and acetonitrile (B), both containing 0.1% formic acid. To achieve separation, the gradient used was: 0-2 min, 5% B; 2-5 min, 12% B, 5-10 min, 12% B, 10-60 min, 40% B. Peaks were detected using UV wavelengths of 210 to 360 nm and fractions corresponding to target compounds were collected into glass tubes. Twelve injections (100 µL each) were performed and fractions from repeated runs were combined and the solvent evaporated using a Speedvac concentrator (Genevac, Suffolk, UK).

3.2. Plant material and metabolite extraction

NWC1283 is one of 16 genotypes that makes up CS/782. CS/782 was planted using winter dormant 20cm cutting in May 2017 at Rothamsted Research (Hertfordshire, UK) (51º48’N, 0º21’W). The previous crop was winter wheat. Soil type within the field is silty clay loam with flints over clay. Planting and agronomy followed conventional SRC best practice. Willows were planted as cuttings using the typical twin-row design at a planting density of 16,667 plants ha^{-1}, establishment year growth received no fertilisers and two pre-emergence herbicides were applied within 10 days of planting pendimethalin (Stomp at 3.3 l/ha) and Isoxaben (Flexidor at 1.0l/ha). Genotypes were planted in unreplicated plots each containing 80 plants (two twin rows of 40).

The winter dormant above ground biomass was harvested in January 2018. Stems were cut 5cm above the ground and immediately chipped in a Petrol Shredder 100mm (4"). Material was stored at -80 ˚C and then freeze-dried. A voucher specimen has been retained and is available on request.

The chips (60 g) were extracted at room temperature, by soaking with 400 ml water:ethanol (4:1) for 16 h. Aliquots were taken for initial metabolite profiling by ^{1}H-NMR and uHPLC-MS and for compound isolation by HPLC.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: NMR chemical shift data of p-hydroxybenzylacetone-β-D-glucopyranoside (**5**), Table S2. NMR chemical shift data of chavicol-α-L-arabinofuranosyl-(1''→6')-β-D-glucopyranoside (**18**) and chavicol--β-D-apiofuranosyl-(1''→6')-β- D-glucopyranoside (**20**)

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